

## MODE OF ACTION OF PROTEIN SYNTHESIS INITIATION FACTOR eIF-1 FROM RABBIT RETICULOCYTES

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### 1. Introduction

Initiation of eukaryotic protein synthesis is mediated by a set of protein factors, purified using predominantly rabbit reticulocytes [1–5] and Krebs II ascites cells [6]. At least 8 initiation factors with  $M_r$  15 000–700 000 could be described. This elaborate work was followed by the functional characterization of the individual factors which provided considerable insight in the specific contribution of each factor during the assembly of an initiation complex ([7–9], reviewed [10]).

eIF-2, eIF-3 and eIF-4C are mainly involved in the binding of the initiator tRNA (Met-tRNA) to a nascent 40 S initiation complex [7,8,10–12], while eIF-1, eIF-4A, eIF-4B and a protein with  $M_r$  24 000 called cap binding protein, participate in the binding of the messenger to this complex. eIF-5 is a 'joining' factor which combines the 40 S · Met-tRNA · mRNA complex to the 60 S ribosomal subunit.

The messenger binding step is particularly interesting since at this stage the selection of a specific messenger occurs and translation of a specific RNA sequence is initiated. Why the process requires the presence of 4 factors is not fully understood [7–10]. eIF-4B [10] and eIF-4E, the cap binding protein [9] seem to be involved in recognizing the cap structure while eIF-4A appears to play a role in the selection of specific messengers [14].

Very little information is available on eIF-1. It is a polypeptide of  $M_r$  15 000 which optimizes the different assay systems such as amino acid incorporation in pH 5 systems and mRNA binding to 80 S initiation complexes with purified initiation factors. The observed requirement, however, is not absolute: a 1.6–2.0-fold stimulation was measured in [7,8,15].

eIF-1 appears to be active with natural mRNAs, but not with AUG; a direct effect on Met-tRNA binding has not been demonstrated [7,8,16].

To further substantiate the role of eIF-1, we have measured its effect in a number of assay systems: Met-tRNA, Val-tRNA and mRNA binding to nascent initiation complexes, methionyl puromycin formation and amino acid incorporation with various mRNAs. We found a 2-fold stimulation by eIF-1 of the Met-tRNA binding, but an almost absolute dependence on the factor of mRNA binding to nascent initiation complexes. The effect of eIF-1 in assays for protein synthesis is the same for capped and non-capped messengers, suggesting that eIF-1 is not involved in the recognition of the cap-structure. A possible mechanism of action is discussed.

### 2. Materials and methods

#### 2.1. Materials

The following components were prepared as in [11,12]: ribosomal subunits, 80 S ribosomes, pH 5 enzymes, hemoglobin 9 S mRNA, reticulocyte lysate, tRNA, [ $^3$ H]Met-tRNA and purified reticulocyte initiation factors. [ $^3$ H]Val-tRNA was prepared essentially as for [ $^3$ H]Met-tRNA in a 100  $\mu$ l system containing 20  $\mu$ Ci [ $^3$ H]valine, 30  $\mu$ M methionine, 400  $\mu$ g pH 5 enzymes and 200  $\mu$ g tRNA under the ionic conditions of initiation complex formation (see below). EMC RNA was a gift from Dr D. P. Leader, VSV RNA was isolated following standard procedures by oligo(dT) cellulose chromatography [11]. The following buffer was used: 20 mM Tris-HCl (pH 7.6), 0.1 mM Na<sub>2</sub>EDTA, 7 mM 2-mercaptoethanol, 40 mM KCl, 80 mM KOAc and 0.6 mM Mg(OAc)<sub>2</sub> (buffer A).

## 2.2. General methods

Gradients of 15–32% (w/w) sucrose in buffer A were prepared as in [11]. Protein concentrations were estimated from the absorbance at 280 nm and 260 nm according to [17].

## 2.3. Labelling of 9 S mRNA

Labelling of mRNA with  $\text{Na}^{125}\text{I}$  was performed as in [18]. In short: 250  $\mu\text{l}$  contained 100 mM KAc-HAc (pH 5.0), 20  $\mu\text{M}$  KI, 100  $\mu\text{Ci}$   $\text{Na}^{125}\text{I}$  (100 Ci/mmol), 0.28 mM  $\text{TiCl}_3$  and 200  $\mu\text{g}$  9 S mRNA. After 15 min at 60°C 3 vol. 50 mM Tris-HCl (pH 9.0), 100 mM NaCl, 1 mM  $\text{Na}_2\text{EDTA}$  and 1 mM  $\text{Na}_2\text{SO}_3$  were added. After 20 min at 60°C, the unreacted iodine was removed by gel-filtration on a Sephadex G-25 column, the mRNA containing fractions were collected, lyophilized and dissolved in  $\text{H}_2\text{O}$ .

## 2.4. Assays

**40 S initiation complex formation:** In 25  $\mu\text{l}$  was contained 20 mM Hepes-KOH (pH 7.6), 1 mM ATP, 0.4 mM GTP, 5 mM creatine phosphate, 1 mM dithiothreitol, 40 mM KCl, 80 mM KOAc, 100  $\mu\text{M}$  spermine, 2 mM  $\text{Mg}(\text{OAc})_2$ , 0.05 unit creatine kinase, 0.4  $\mu\text{M}$  Met-tRNA, 0.1  $A_{260}$  unit of 40 S subunits, 0.2  $A_{260}$  unit 60 S subunits, 0.5  $\mu\text{g}$  hemoglobin mRNA, 0.3  $\mu\text{g}$  eIF-1, 1  $\mu\text{g}$  eIF-2, 3.5  $\mu\text{g}$  eIF-3, 1  $\mu\text{g}$  eIF-4A, 1  $\mu\text{g}$  eIF-4B and 0.16  $\mu\text{g}$  eIF-4C.

The mixtures were incubated at 37°C for 15 min, layered on sucrose gradients and centrifuged for 90 min (80 S initiation complexes) or 3 h (40 S initiation complexes) at 50 000 rev./min. Fractions of 300  $\mu\text{l}$  were collected and counted as in [12].

**80 S Initiation complex formation:** This assay was performed as for 40 S initiation complex formation, except that 0.5  $\mu\text{g}$  of eIF-5 was added.

**Protein synthesis in 'pH 5' systems:** A 25  $\mu\text{l}$  incubation mixture composed as for 80 S initiation complex formation was supplemented with 50  $\mu\text{M}$  each of 19 unlabelled amino acids, 5  $\mu\text{Ci}$   $^{35}\text{S}$ -methionine (800 Ci/mmol) and 20  $\mu\text{g}$  pH 5 enzymes. The Met-tRNA was replaced by 0.03  $A_{260}$  unit of tRNA. After 60 min at 37°C, the  $^{35}\text{S}$ -methionine incorporation into protein was measured as in [12].

**Val-tRNA binding to 80 S ribosomes:** This assay was performed as for 80 S initiation complex formation, except that [ $^3\text{H}$ ]Val-tRNA (0.4  $\mu\text{M}$ ) and 1.5  $\mu\text{g}$  EF-1, purified as in [19], were added.

**Methionyl puromycin formation:** This assay was done as in [11].

## 3. Results

### 3.1. The effect of eIF-1 in pH 5 assay systems for protein synthesis

The dependence on eIF-1 in the initiation of protein synthesis directed by globin mRNA was measured in [1,8]. A 1.5–2.0-fold stimulation by eIF-1 of globin synthesis in a 'pH-5' system utilizing purified initiation factors was reported and eIF-1 suggested to be a contaminant of the pH 5 enzyme preparation [1,8]. In our hands, the dependence on eIF-1 was much more pronounced. As shown in fig.1, a 5-fold stimulation of methionine incorporation into globin was observed in the presence of 7 highly purified initiation factors. The dependence on eIF-1 in our assay system was in fact comparable to that observed for eIF-2, eIF-3 and other essential factors [12]. Apparently, our pH 5 fraction was virtually free of eIF-1.

The question could be raised whether eIF-1 is analogous to or contaminated with the cap-binding protein ( $M_r$  24 000), purified and partially characterized in [9]. However, a number of observations argue against this possibility:

- We could not replace eIF-1 by cap-binding protein, purified according to [9], in the experiment of fig.1 (results not shown);
- We checked whether eIF-1 could discriminate

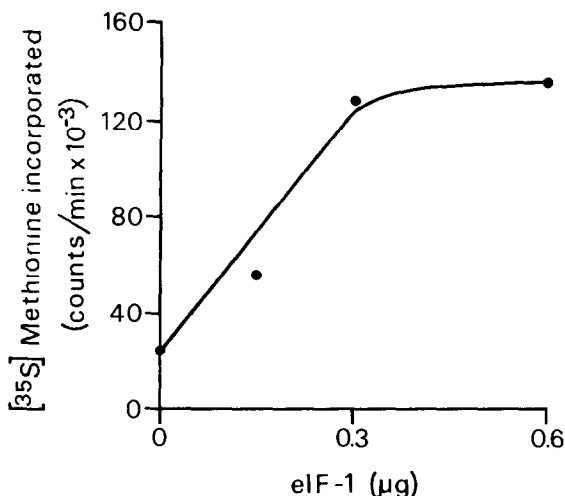


Fig.1. Effect of eIF-1 on hemoglobin mRNA directed protein synthesis. Protein synthesis was measured in a 'pH 5' assay as in section 2. A blank incorporation of 17 000 cpm obtained in the absence of initiation factors has been subtracted.

between capped and non-capped messengers, such as VSV mRNA (capped) and EMC RNA (non-capped, [20]).

The RNAs were translated in the pH 5 system and the dependence on eIF-1 was determined. The results of such an experiment are shown in fig.2. It is clear that the stimulation caused by the addition of eIF-1 is approximately equal in both cases, which suggests that the cap-structure does not play a crucial role in the functioning of eIF-1 and that eIF-1 and cap-binding protein are two different entities.

### 3.2. The effect of eIF-1 on 40 S and 80 S initiation complex formation

It is not entirely obvious at which particular step during the assembly of an initiation complex eIF-1 displays its activity. Literature data show some effect of the factor on the binding of Met-tRNA and mRNA to 40 S and 80 S initiation complexes [1,7,15,16]. However, the effects are small and information on the precise mode of action at the molecular level is lacking (see section 1).

We have repeated therefore, part of the studies on Met-tRNA and mRNA binding using a slightly different approach: instead of studying initiation com-

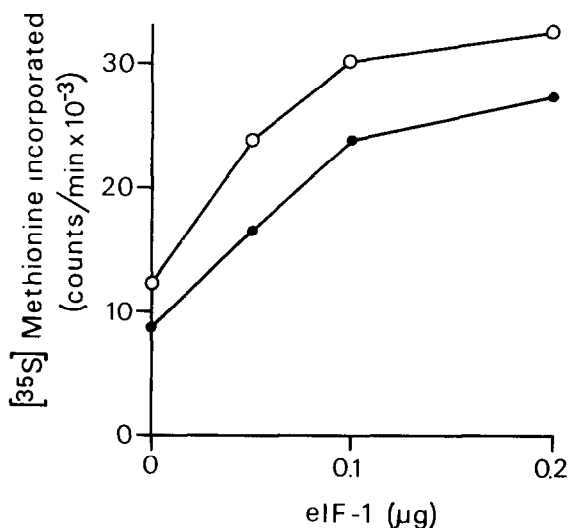


Fig.2. Effect of eIF-1 on the translation of EMC and VSV mRNA. Protein synthesis in a 10  $\mu$ l mixture was measured in a pH 5' assay as in section 2. Aliquots of 5  $\mu$ l were taken to measure the incorporated methionine. A blank incorporation of 11 000 cpm obtained in the absence of mRNA has been subtracted. Of each mRNA 0.4  $\mu$ g was tested: (○—○) VSV mRNA; (●—●) EMC mRNA.

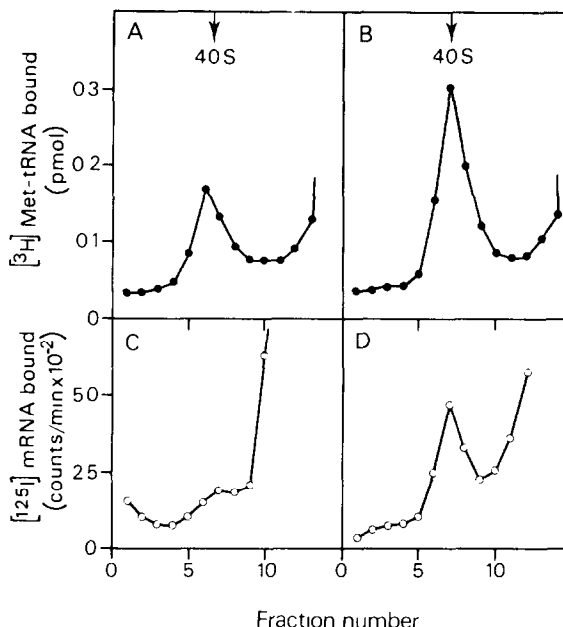


Fig.3. The effect of eIF-1 on 40 S initiation complex formation. 40 S Initiation complex formation was done as in section 2. (A,C) minus eIF-1; (B,D) plus eIF-1. (●—●) [<sup>3</sup>H]-Met-tRNA; (○—○) [<sup>125</sup>I]-mRNA.

plex formation with separated subunits, whole 80 S ribosomes were used (or formed by preincubation of 40 S and 60 S ribosomal subunits). This procedure creates an assay system with more physiological relevance, since the dissociation of 80 S ribosomes is thought to occur *in vivo* as the first step in the initiation of protein synthesis [21]. 40 S initiation complex formation can be studied this way simply by omitting eIF-5 from the incubation mixtures.

The dependence of [<sup>3</sup>H]Met-tRNA and [<sup>125</sup>I]-globin mRNA binding on eIF-1 to 40 S and 80 S initiation complexes was examined using this approach. The results are shown in fig.3 (40 S initiation complex formation) and fig.4 (80 S initiation complex formation). Addition of eIF-1 to the reaction mixtures resulted in both cases in a 2-fold stimulation of Met-tRNA binding (fig.3A,B, fig.4A,B). This effect of eIF-1 on Met-tRNA binding could also be obtained in the complete absence of mRNA and eIF-4A and eIF-4B (not shown). The dependence of mRNA binding on eIF-1 is much stricter. As shown in fig.3C vs 3D for 40 S and in fig.4C vs 4D for 80 S initiation complex formation omission of eIF-1 virtually abolished the mRNA binding. In fact, the need for eIF-1

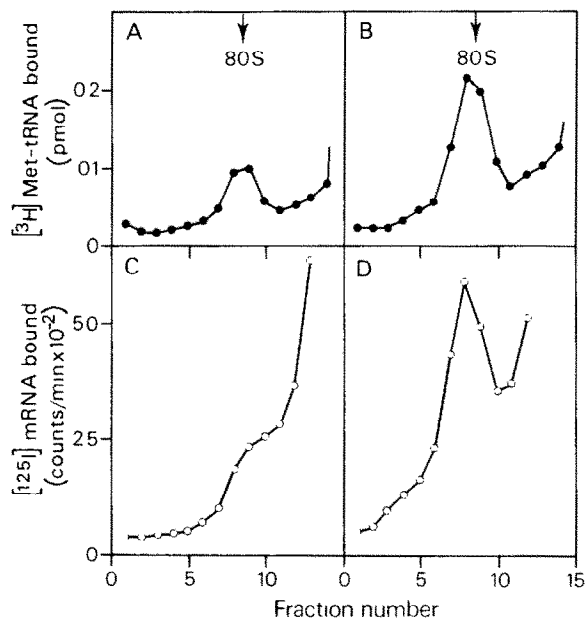


Fig.4. Effect of eIF-1 on 80 S initiation complex formation. 80 S initiation complex formation was done as in section 2. (A,C) minus eIF-1, (B,D) plus eIF-1. (●—●) [ $^3\text{H}$ ]Met-tRNA, (○—○) [ $^{125}\text{I}$ ]mRNA.

in this reaction parallels the need for eIF-1 in the pH 5 system of fig.1.

The effect of eIF-1 on the binding of globin mRNA is further illustrated in the experiment of fig.5 in which the binding of Val-tRNA to 80 S initiation complexes was measured. Valine is the second amino acid of both  $\alpha$  and  $\beta$  globin [22] and the presence of Val-tRNA on the ribosome should depend entirely on the preceding binding of globin messenger. Fig.5 shows, indeed, that the effect of eIF-1 on mRNA binding (see fig.3,4) enhances concomitantly the bind-

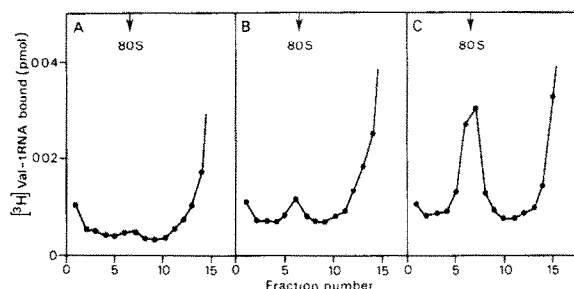


Fig.5. [ $^3\text{H}$ ]Val-tRNA binding to 80 S initiation complexes. [ $^3\text{H}$ ]Val-tRNA binding was done as in section 2. (A) minus eIF-1; (B) minus mRNA; (C) complete.

ing of Val-tRNA (fig.5A,C). The omission of eIF-1 from the incubation mixture abolishes Val-tRNA binding to the same extent as the omission of mRNA (fig.5B).

The results of fig.3–5 indicate that eIF-1 behaves differently from the other initiation factors involved in mRNA recognition (eIF-4A, eIF-4B and the  $M_r$  24 000 protein), since no effect on the binding of Met-tRNA by these factors could be demonstrated (not shown, [8,9]).

### 3.3. The effect of eIF-1 on methionyl-puromycin formation

The synthesis of methionyl puromycin may be used as a check on the physiological relevance of Met-tRNA binding to 40 S and 80 S initiation complexes as observed by sucrose gradient analysis. We have determined, therefore, the effect of eIF-1 in this reaction performed either in the absence or presence of AUG. The results are shown in table 1.

In the absence of AUG a 3-fold stimulation of the methionyl-puromycin synthesis was observed upon addition of eIF-1, in good agreement with the results of fig.3,4. Surprisingly, in the presence of AUG, the stimulation by eIF-1 was no longer detectable, which suggests that the stimulatory effects of AUG and eIF-1 originate from a similar molecular event. Apparently, the increase in stability of Met-tRNA binding which is thought to arise from the codon–anticodon interaction, can also be provided by eIF-1. The stimulation by eIF-1 was not due to RNA contamination, since eIF-1 treated with *N*-ethylmaleimide (NEM) was virtually inactive.

Table 1  
Effect of eIF-1 on the methionyl puromycin formation

Addition	[ $^3\text{H}$ ]Methionyl-puromycin formed (pmol)	
	–AUG	+AUG
None	1.0	3.3
eIF-1	3.3	3.1
NEM–eIF-1	1.2	3.8
NEM buffer	1.0	3.3

Methionyl puromycin formation was done as in section 2. NEM–eIF-1 was prepared by incubating eIF-1 for 30 min at 37°C with 10 mM NEM. The reaction was stopped by adding 100 mM dithiothreitol. As a control the same procedure was followed without eIF-1 (NEM buffer, see table). The effect of the treatment of eIF-1 done in the absence of NEM was negligible

#### 4. Discussion

This paper describes the effect of eIF-1 in several model assay systems for the initiation of protein synthesis, with particular emphasis on its role in the binding of mRNA and Met-tRNA. It appears that eIF-1 is an essential factor, without which no mRNA binding occurs and of equal importance in the initiation process as better described factors, i.e., eIF-2, eIF-3, eIF-4C, eIF-5.

The differential effect of eIF-1 on Met-tRNA and mRNA binding is intriguing. The stimulatory effect by eIF-1 on Met-tRNA binding to 40 S (see fig.3) could not be explained by an increase in the dissociation level of the 80 S ribosomes utilized, since no effect of eIF-1 on the dissociation of 80 S ribosomes was measured (not shown). Instead, it appears that eIF-1 has a stabilizing effect on the binding of Met-tRNA similar to the effect of AUG (fig.3,4, table 1). An eIF-1 induced re-positioning of the Met-tRNA, facilitating the codon-anticodon interaction, might be the basis for the stabilized binding. It is feasible, that a proper alignment of Met-tRNA is obligatory for stable binding of mRNA. Thus, the primary action of eIF-1 appears to be on Met-tRNA binding, in agreement with the finding that the same stimulatory effect of the factor on Met-tRNA binding could be measured in the complete absence of mRNA, eIF-4A or eIF-4B.

In the model proposed [23], 40 S ribosomes migrate along the messenger till they reach the initiation codon. eIF-1 does not seem to be involved in the primary recognition of messenger and ribosome (see fig.2), but may turn out to be the factor which stops the 40 S movement by coordinating the codon-anticodon interaction. It is not known at which stage the factor is present on nascent initiation complexes since, unfortunately, a method of radiochemical labelling which does not destroy its biological activity is not available [8].

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